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Description of a Male Urogenital Papilla in the California Grunion, *Leuresthes tenuis*, a Beach-spawning Marine Silverside Fish

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The California grunion, *Leuresthes tenuis* (Ayres, 1860), like its sister species, the Gulf grunion, *L. sardina* (Jenkins & Evermann, 1889), engages in unusual reproductive behavior in which males and females aggregate on sandy beaches to spawn (Thompson 1919; Walker 1949, 1952; Thomson and Muench 1976; Martin 2015). Spawning by California grunion occurs at night during semilunar spring high tides in March-August, from Magdalena Bay, Baja California, Mexico, to Tomales Bay, California (Walker 1952; Moffatt and Thomson 1975; Roberts et al. 2007; Martin et al. 2013). Females deposit eggs ~5-10 cm deep in the sand while males release sperm as they surround females at the sand surface. The externally fertilized eggs develop within the sand until competent to hatch. Hatching is triggered when the embryos are washed out of the sand during a subsequent spring high tide series, and the swimming larvae are washed into the ocean (Thompson 1919; Griem and Martin 2000; Martin et al. 2011). While extracting gametes for other studies, we noticed a structure associated with gamete release in California grunion males, but not females. In this study, we used dissections, magnetic resonance imaging (MRI), paraffin histology and light microscopy to characterize that structure as a muscular urogenital papilla, a sexually dimorphic character that can be used to distinguish the sexes noninvasively. We also propose a function for the urogenital papilla in external fertilization in this beach-spawning species.

Adult California grunion were collected during spawning runs on Cabrillo Beach, San Pedro, CA (GPS coordinates: 33°42′33″N, 118°16′59″W), and Doheny State Beach, Dana Point, CA (33°27′43″N, 117°41′14″W), under California Department of Fish and Wildlife Scientific Collecting Permits (SC-3211, SC-4783, SC-10567, and SC-10585). Whole fish were placed on ice or dry ice, or euthanized in tricaine methanesulfonate (MS-222; 0.2 g L^{-1} of seawater) and fixed in 10% phosphate-buffered formalin, and then transported to California State University Fullerton (CSUF). Iced or frozen and thawed whole males (N = 6) were dissected to examine the urogenital papilla and its relationship with other organs, and several females were also dissected to examine the ovary and oviduct. One fixed male and one female were prepared for MRI, and segments of other fixed individuals (15 males and 8 females) were processd for histology. All work was approved by the CSUF Institutional Animal Care and Use Committee under protocols 08-R-07, 11-R-07, 14-R-07, and 17-R-06.

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The MRI image data were acquired at the Keck Center for Functional MRI at the University of California San Diego on a 7T (300 MHz, 20 cm bore) small animal imaging system (Bruker Biospec Avance II, Bruker AXS Inc., Madison, WI) fitted with a 72-mm inner diameter quadrature RF volume coil (Bruker Biospin GmbH, Ettlingen, Germany). All images were acquired with a standard T1-weighted 3D fast spoiled gradient recalled echo acquisition pulse sequence with images collected in the transverse plane. The entire body of a male specimen was scanned at an isotropic voxel resolution of 100 μm using the following pulse sequence parameters: 15°FA, 26.6 ms TR, 13.2 ms TE, 300 kHz bandwidth, and 3 averages. An additional higher resolution scan of the midbody region containing the urogenital papilla was then acquired at 80 μm isotropic resolution (with 6 averages). The corresponding midbody region of a female was also scanned at 80 μm using the same pulse sequence parameters as the male. The MRI data are freely available upon request in the original 32-bit integer format or other standard medical imaging formats at the Digital Fish Library (http://www.digitalfishlibrary.org/library/ViewSpecies.php?id=334). MRI image data of the male in NIfTI format were segmented using ITKSnap (ver. 3.6.0, http://www.itksnap.org/pmwiki/pmwiki.php) and smoothed and rendered in 3D using Blender (ver. 2.79b, https://www.blender.org), to reconstruct the wall of the urogenital papilla, the distal intestine to the anus, the sperm duct from the two testes to the genital pore, and the mesonephric urinary duct from the trunk kidney to the urinary pore.

The histology samples were rinsed in phosphate buffer, dehydrated in a graded ethanol series, cleared with xylenes, and infiltrated and embedded in paraffin wax. Tissue blocks were sectioned with a rotary microtome and 5-μm-thick sections were mounted on glass slides and stained either with hematoxlyn and eosin or with Mallory's trichrome (Humason 1979). Transverse sections and sagittal sections of males containing the urogenital papilla, and of females in the corresponding body regions, were examined. Sections of isolated papillae dissected from formalin-fixed males were also prepared and examined.

Normally, in live male grunion collected while spawning on shore, the urogenital papilla does not protrude from the body. When pressure was applied externally to the ventral side of a male grunion, the papilla would project externally (Fig. 1A) and sperm could be ejected a considerable distance (more than one body length). Applying pressure to the ventral surface of female grunion released unfertilized eggs but did not result in protrusion of a corresponding structure. A lateral view of a male grunion that was partially dissected (Fig. 1B) shows the urogenital papilla to be a vascularized, muscular structure between the anus and anal fin. In males that were examined under a dissecting microscope, application of pressure caused sperm to flow from the sperm duct within the papilla and fecal material to exit via a separate anal opening. Injections of ink and MRI imaging confirmed that the sperm duct within the urogenital papilla connects with the testes. The MRI and histological analyses revealed additional details about the structure of the papilla, indicating that it contains both the sperm duct and the mesonephric urinary duct, and how it differs from the oviduct of the female. MRI images of longitudinal sections of the male (Fig. 1C, D) show the muscular wall of the urogenital papilla and its position relative to the intestine, testes, and urinary duct. The 3D segmentation of the MRI data (Fig. 1E) traces the distal intestine to the anus, the posterior region of the testes to the sperm duct, and the urinary duct descending from the posterior region of the trunk kidney into the urogenital papilla in a position posterior and dorsal to the sperm duct. Transverse sections of males provide more details of the papilla's structure and the relative positions of the sperm duct, urinary duct, distal intestine, and anus (Fig. 2). In more anterior sections (Fig. 2A, D, E), the

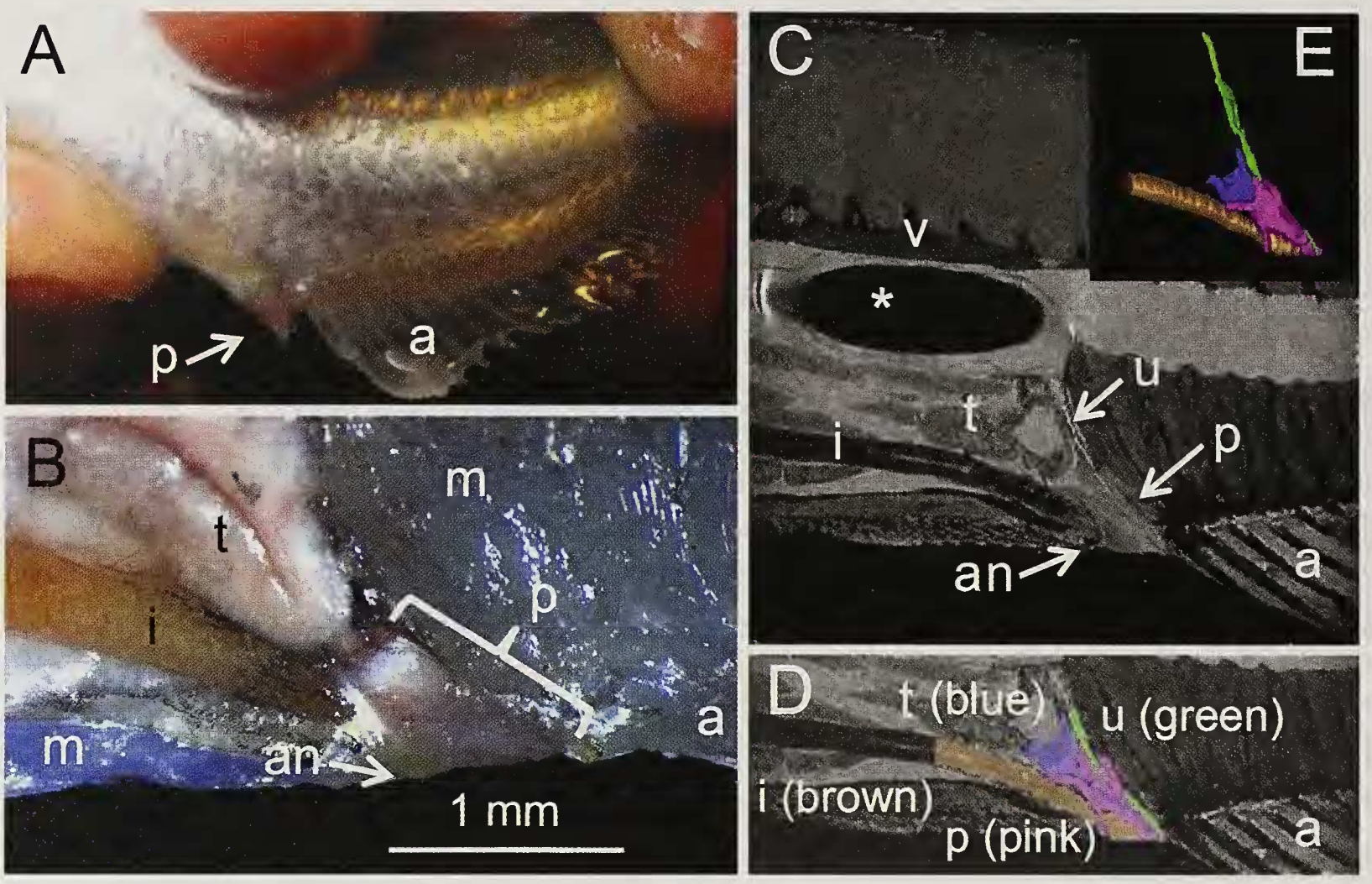

Fig. 1. Urogenital papilla in male California grunion, *Leuresthes tenuis*. A. Lateral view of the midbody region of a male with urogenital papilla (p) protruding just anterior to the anal fin (a). Anterior of fish is to the left. B. Lateral view of partially dissected male grunion showing vascularized, muscular urogenital papilla (p), located between the intestine (i) and anus (an) and the anal fin (a), and through which sperm is released from the testes (t). m = body wall and anal fin skeletal muscle. C. MRI longitudinal section of a male grunion, showing the internal position of the muscular wall of the urogenital papilla relative to the distal intestine (i) and anus (an), its connection to the testes (t), and the urinary duct (u) from the trunk kidney into the urogenital papilla; v = vertebral column. The dark area indicated by * is an artifact of the MRI scan in the region containing the gas bladder. In D, the color-coded segmentation is overlain over part of the section in C, with the wall of the urogenital papilla (pink), the sperm duct traced from the testes (blue), the urinary duct traced from the trunk kidney through the urogenital papilla (green), and part of the distal intestine (brown). E shows the 3D rendering of the urogenital papilla from all of the MRI data; segmentation colors as in D.

urinary duct is separate from the papilla but joins it more posteriorly at the transverse position of the anal opening (Fig. 2B, F).

Comparison of longitudinal, transverse, and sagittal sections in the region containing the urogenital papilla in males (Fig. 1C, 2A, B, D-F, 3F, G) with corresponding regions in females (Fig. 2C, 3A-E) indicates that the urogenital papilla in males is larger and more muscular than the oviduct. When sections were viewed at higher magnification (e.g., Fig. 2E, F, 4B), sperm were visible within the sperm duct of the papilla, and tissues comprising the papilla could be identified. The asymmetrical muscular wall of the urogenital papilla (Fig. 2F, 3F, 4A) was found to contain, in addition to smooth muscle, striated skeletal muscle fibers (Fig. 4C) which were absent in the wall of the oviduct. The presence of skeletal muscle in the wall of the urogenital papilla, but not the oviduct, was confirmed in sections stained with Mallory's trichrome (not shown).

Taken together, our data show that males of the California grunion have a urogenital papilla, dorsal to the intestine and terminating posterior to the anus, a morphological feature not present in females. The females have a less muscular oviduct through which

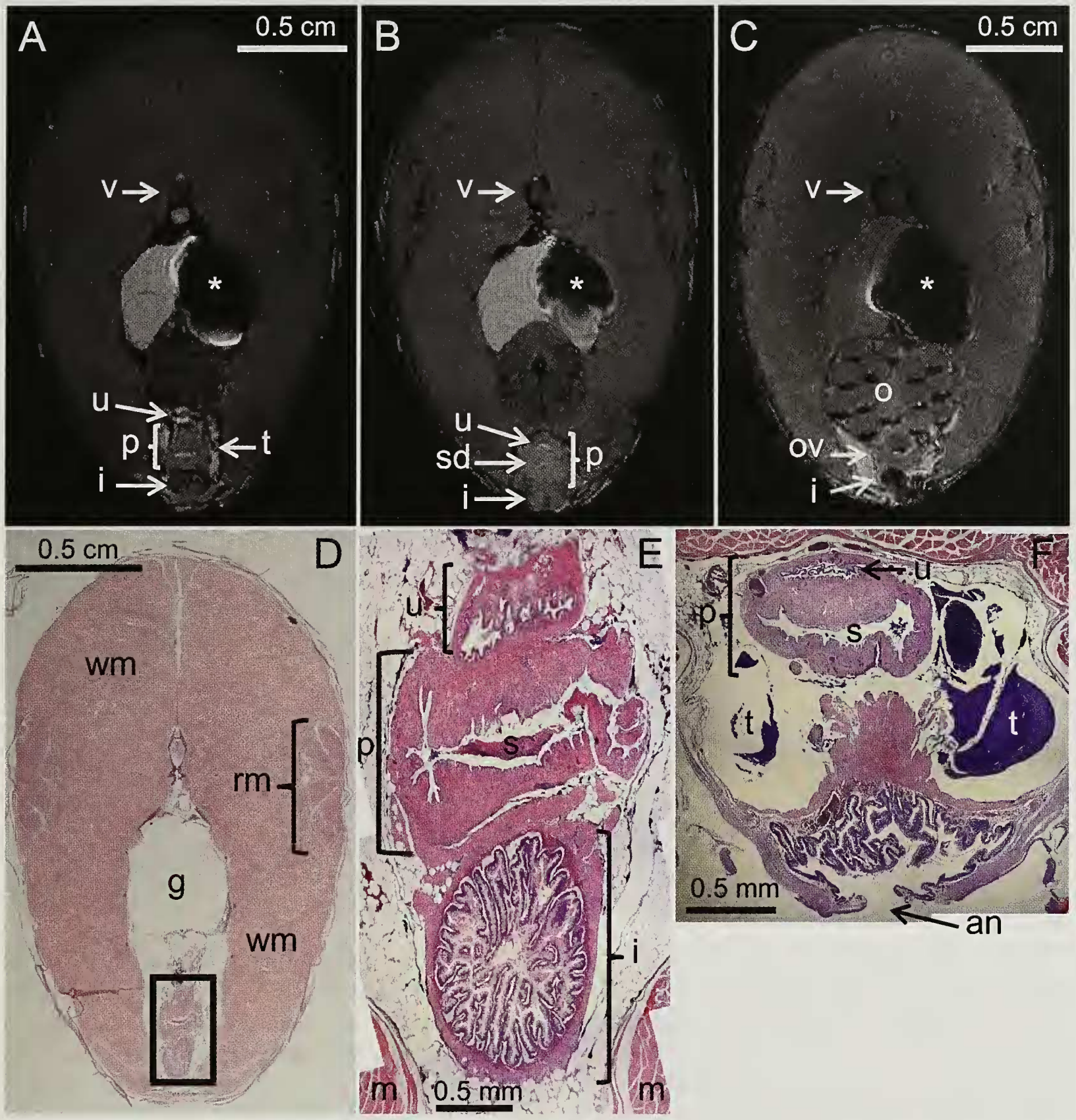

Fig. 2. A, B. MRI images of transverse sections of a male grunion at two positions along the body in the region containing the urogenital papilla (p), with A more anterior than B. Scale bar in A also applies to B. In A, the urinary duct (u) is separate from the urogenital papilla, which contains the sperm duct (sd), whereas in B the urinary duct (u) is within the urogenital papilla, which is dorsal to the intestine (i); v = vertebra, t = testis; the dark areas (*) are an artifact of the MRI scan due to the presence of the gas bladder. C. MRI images of transverse sections of female grunion at a position similar to A, with the ovary containing oocytes (o) and oviduct (ov) dorsal to the intestine (i). D. Transverse histological section of a male in the region corresponding to A, stained with hematoxylin and eosin. Gas bladder (g), slow-oxidative (rm) and fast-oxidative (wm) locomotor muscle are indicated. E. Higher magnification of the boxed area in D with the urinary duct dorsal to the papilla containing the sperm duct (s), which is dorsal to the intestine (i); m = skeletal muscle of the body wall. F. Urogenital papilla in the region of the body near that shown in B, with the urinary duct within the urogenital papilla, and the intestine ending at the anal opening (an); t = paired testes.

oocytes pass as they are deposited in the sand and then fertilized by males during beach spawning. During oviposition, individual females are partially buried in wet sand and are surrounded by multiple males at the sand surface (Thompson 1919; Walker 1952; personal observations). Based on genetic analysis of egg clutches in the sand, which contain

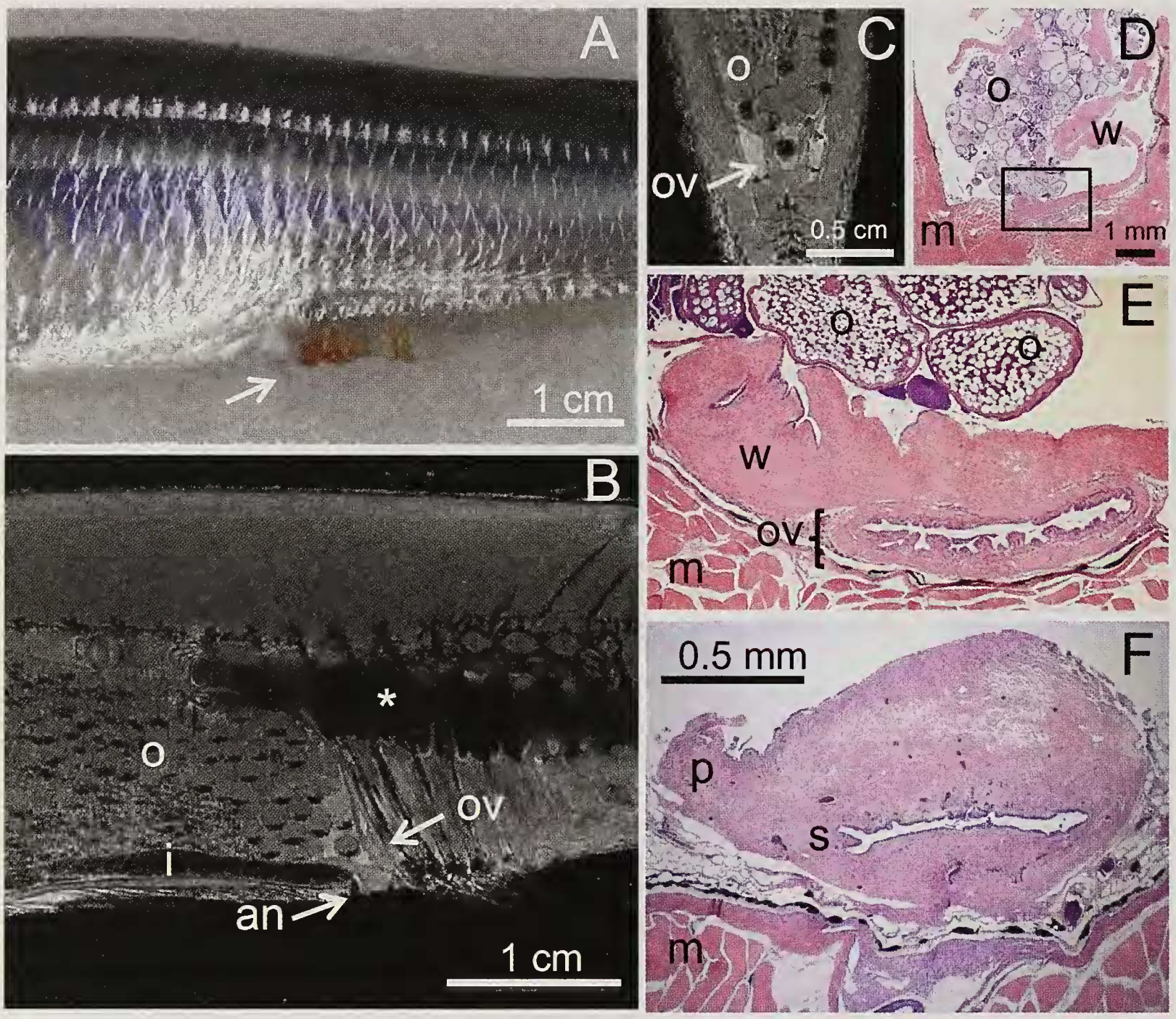

Fig. 3. A. Lateral view of the midbody region of a female California grunion. Arrow points to the genital opening through which oocytes are released, with no protruding structure evident. In A and B, anterior of fish is to the left. B. MRI image of a longitudinal section of a female grunion, showing part of the ovary containing oocytes (o), the oviduct (ov), the distal intestine (i) and the anus (an). The dark area indicated by * is an artifact of the MRI scan in the region containing the gas bladder. C. MRI image of female sagittal section showing the ovary (o) and an oocyte in the oviduct (ov). In C-F, the top of the image is anterior in the fish. D. Histological sagittal section similar to C, stained with hematoxylin and eosin, showing oocytes (o) within the ovary wall (w), a section through the oviduct (box magnified in E), and the surrounding body musculature (m). E. Higher magnification of the boxed area in D, showing the ovary wall (w) and oviduct (ov) wall which contains smooth but not striated muscle. F. Sagittal section of the male genital papilla in a similar position as in the female in E. Scale bar in F also applies to E.

approximately 1000-3000 eggs (Walker 1952; Martin and Carter 2013), up to nine males may fertilize the eggs of one female (Byrne and Avise 2009). There is, therefore, some degree of sperm competition in this species, and we propose that the urogenital papilla allows males to release sperm in a more directed, high-pressure stream to increase the chances of successful fertilization of eggs that a female has deposited in the sand.

Genital and urogenital papillae have been described in numerous fish species, including other members of the Series Atherinomorpha and Order Atheriniformes, of which the California grunion is a member (Dyer and Chernoff 1986; Parenti 2005; Evans and Meisner 2009; Fishelson et al. 2013). In many of these other atherinid fishes, including members of the genus *Labidesthes*, the only other member of the Family Atherinopsidae in which a male urogenital papilla has been described, the papilla is used as an intromittent

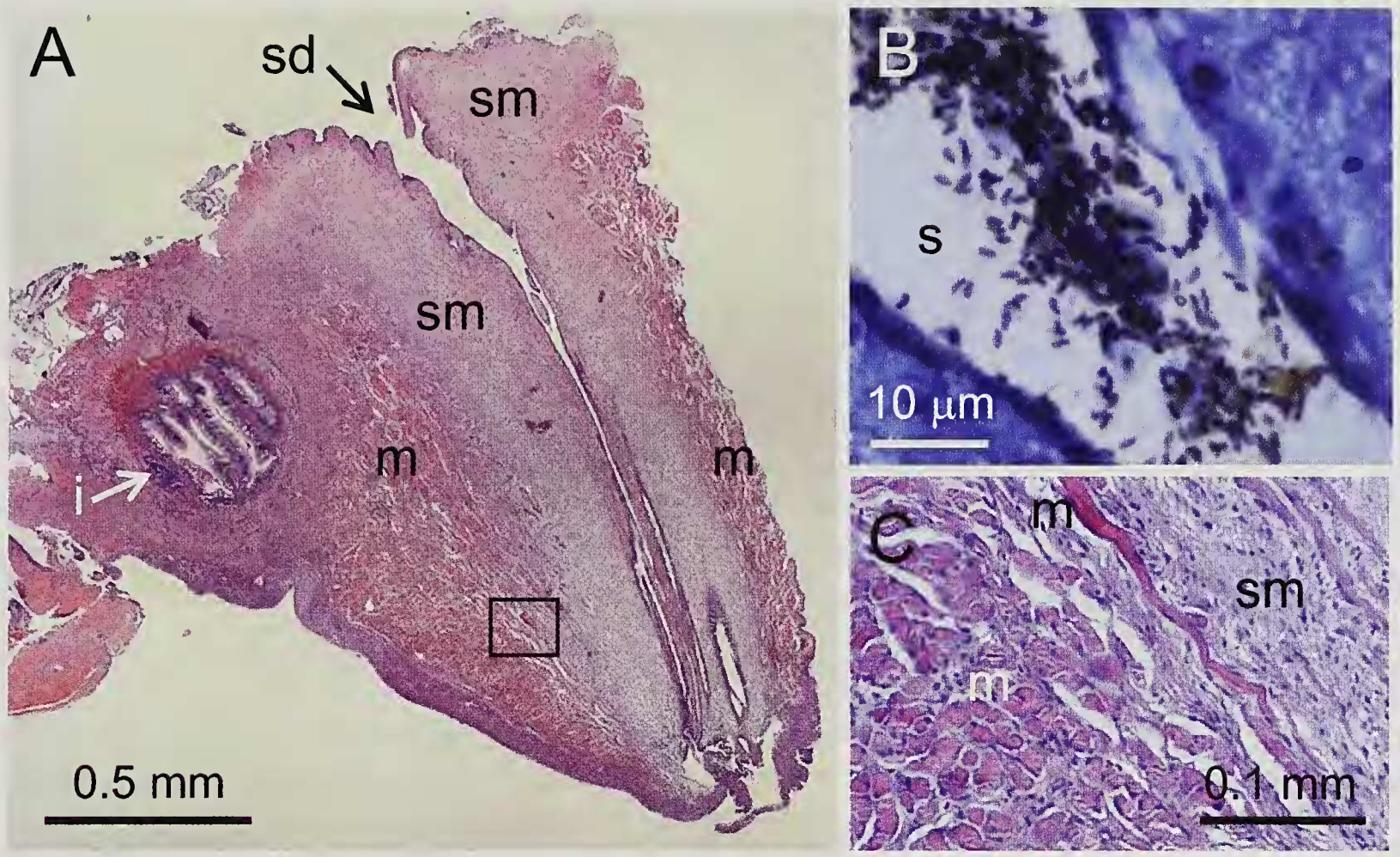

Fig. 4. A. Longitudinal section of whole isolated urogenital papilla and a section of the intestine (i) near the anus, stained with hematoxylin and eosin. Anterior of fish is to the left. The sperm duct (sd) is surrounded by layers of smooth muscle (sm) and striated skeletal muscle (m). B. Higher magnification of part of the sperm duct, showing sperm (s) within the lumen. C. Higher magnification of the black boxed area in A, showing striated skeletal muscle fibers within the urogenital papilla wall.

organ for internal fertilization (Grier et al. 1990; Werneke and Armbruster 2015). In several teleost species, females have a genital papilla that is used as an ovipositor to release oocytes that are then fertilized externally. There are numerous other teleost species in which males release sperm via a urogenital papilla to fertilize eggs externally, including many gobies and blennies (e.g., Har 2000; Ferreira et al. 2010; Fishelson et al. 2013). In the cichlid *Herichthys minckleyi*, "sneaker" males use the urogenital papilla to externally fertilize eggs while another male and female are involved in courtship and oviposition (Oldfield et al. 2015). Rasotto and Shapiro (1998) described the urogenital papilla in males of the bluehead wrasse, *Thalassoma bifasciatum* (Bloch, 1791), which is histologically similar to that of the California grunion and is used in external fertilization during group spawning in this coral-reef sequential hermaphrodite. Rasotto and Shapiro (1998) proposed that the muscular urogenital papilla of male bluehead wrasse allows control of sperm release for multiple, sequential spawning events in a given day. It is possible that the urogenital papilla in the California grunion serves a similar function by limiting sperm release so that males can return to the beach and spawn on the same night or on sequential nights of a spring tide series. If males but not females spawn multiple times during a tide series, this behavior may explain the male-biased sex ratio reported in California grunion samples collected during a given night's spawning event (Thompson 1919; Walker 1949; Santos et al. 2018) and increase a male's chances to fertilize the eggs of more than one female.

To our knowledge, the present study is the first description of a sexually dimorphic characteristic, the urogenital papilla, that can be used to distinguish male and female California grunion externally, and the first study to apply MRI to characterize the three-dimensional structure of a urogenital papilla in any fish species.

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